Decline of DNA Damage and Other Biomarkers in Peripheral Blood following Smoking Cessation[1]

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Abstract

Serial samples from 40 heavy smokers (≥1 pack/day for ≥1 year) enrolled in a smoking cessation program were assayed for cotinine, polycyclic aromatic hydrocarbon (PAH)-DNA, 4-aminobiphenyl-hemoglobin (4-ABP-Hb) adducts, and glycophorin A (GPA) mutations. Blood samples were taken while subjects were smoking, and 10 weeks and 8 and 14 months after quitting. Cotinine was used to assess compliance with the cessation protocol. A significant reduction in mean PAH-DNA and 4-ABP-Hb adducts was observed after cessation in all persons who were cotinine-verified quitters (≤25 ng/ml) for ≥8 months ($P < 0.05$). Neither the GPA N/ϕ nor the GPA N/N mutation V_f was significantly reduced after smoking cessation, but results are limited by the small number ($n = 18$) of heterozygous individuals studied.

The substantial reduction (50–75%) in PAH-DNA and 4-ABP-Hb adduct levels after quitting indicates these carcinogen adducts are reflective of smoking. Passive exposure to smoke at home was significantly associated with PAH-DNA adducts in active smokers and in ex-smokers 10 weeks after quitting ($P < 0.01$). The estimated half-life of the PAH-DNA adducts in leukocytes is 9–13 weeks by inspection of the mean biomarker levels from baseline and 10 weeks sample and 23 (95% confidence interval, 10–36 weeks) using a linear regression model that adjusted for background. Women had higher levels of 4-ABP-Hb adducts at baseline and after smoking cessation after adjustment for amount of smoking, suggesting that women may be more susceptible to carcinogenic exposures. For 4-ABP-hemoglobin adducts, the estimated half-life is 7–9 weeks from inspection of the means, which is consistent with the lifetime of hemoglobin, as compared with 12 weeks (95% confidence interval, 10–14 weeks) using a regression model that adjusted for background. The variability in the confidence intervals for the regression half-lives illustrates that some individuals may be less efficient in eliminating genetic damage than others.

Thus, PAH-DNA and 4-ABP-Hb adducts can be useful as intermediate biomarkers in intervention programs and in identifying persons who may be at increased risk of cancer from exposure to cigarette smoke due to high levels of carcinogen binding.

Introduction

Cigarette smoke is a complex aerosol of more than 4000 chemicals, of which 43 are known carcinogens (1). Included in the toxic mixture are a variety of compounds including nicotine, 4-ABP[3] (an aromatic amine), and BaP (a PAH; Refs. 2, 3). BaP and 4-ABP, both human carcinogens, have been shown to bind covalently to cellular macromolecules *in vivo* (4–7).

BaP is generated by incomplete combustion and has been detected in cigarette smoke (400–800 ng/day for 20 cigarettes); charcoal-broiled, smoked, and grilled meats (10,000 ng/200-g steak); ambient air (9–40 ng/day); and drinking water (1 ng/day; Refs. 8, 9). Numerous previous studies have attempted to assess the effect of smoking on PAH-DNA adduct formation in total peripheral leukocytes, but the results have been inconsistent between studies. Several studies of leukocyte DNA have shown an association between PAH-DNA adduct levels and smoking measured by ELISA (10, 11). Other studies have not been able to demonstrate a statistically significant relationship between adducts and smoking by either ELISA or ^{32}P postlabeling (12–16), although higher levels have been observed in healthy smokers than in nonsmokers (16). However, recent studies have demonstrated an association between smoking and PAH adducts by ^{32}P postlabeling and ELISA using the longer-lived lymphocyte/monocyte fraction of the WBC, supporting their potential to reflect smoking-related damage (17, 18).

4-ABP is a rodent mammary carcinogen (8) and a human bladder carcinogen found in cigarette smoke, dyes, and in occupational settings (19, 20). The majority of nonoccupational human exposure results from active and passive smoking. 4-ABP-Hb adducts have been significantly correlated with recent smoking and smoking status (16, 21, 22) and have been used to measure passive exposure to cigarette smoke (23, 24).

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[3] The abbreviations used are: 4-ABP, 4-aminobiphenyl; 4-ABP-Hb, 4-ABP-hemoglobin; BaP, benzo(*a*)pyrene; PAH, polycyclic aromatic hydrocarbon; V_f, variant frequency; ETS, environmental tobacco smoke; CI, confidence interval; BPDE-I, *r*-7,*t*-8-dihydroxy-*t*-9,10-epoxy-7,8,9,10,-tetrahydrobenzo[a]pyrene; PFPA, pentafluoroproprionic anhydride.

In a prior study, 4-ABP-Hb adducts levels declined by 72% 9–11 weeks after quitting smoking, indicating that Hb adducts reflect active smoking exposure (20).

The GPA *in vivo* somatic cell mutation assay detects and quantifies variant erythrocytes resulting from inactivating mutations at the *GPA* locus. Dramatic increases in hemizygous GPA V_f have been documented in subjects with chromosome instability cancer-prone syndromes (25–27), populations exposed to whole body ionizing radiation (28–31), and in subjects treated with mutagenic chemotherapeutics (32, 33). Elevated GPA V_fs have also been observed in active smokers compared to matched groups of nonsmokers among occupationally exposed workers (34). However, data are very limited on the effect of smoking alone on GPA N/0 mutations. GPA N/0 variants were postulated to be increased in smokers because the null mutation reflects small deletions and point mutations, the type of damage usually caused by BaP. GPA N/N mutation is generally a result of large deletions and chromosome duplication and was not expected to increase with smoking exposure.

Because cotinine, the major metabolite of nicotine, is highly smoking specific and supplies a measure of recent nicotine intake over several days, it was measured in plasma to assess compliance with the cessation protocol (35). The half-life of cotinine is approximately 15 to 40 h in smokers and nonsmokers (35). The plasma concentration of cotinine for active smokers generally ranges from 200 to 500 ng/ml, with values as high as 1000 ng/ml (35, 36). Nonsmokers exposed to passive smoke typically have plasma cotinine values <15 ng/ml (24), although cutoff points up to 42 ng/ml cotinine in serum/plasma have been used to define smoking status (37).

The present study involves heavy smokers (≥20 cigarettes/day) self-selected into a smoking cessation program. We have attempted to assess the contribution of active and passive smoking and dietary exposure to biomarkers by serially sampling subjects before and after removal of the active exposure, and by using questionnaire variables to predict biomarker levels. In this study, each individual acted as his/her own control, eliminating the problem of noncomparable controls and reducing the likelihood that differences between individuals might overshadow the effect of smoking.

The goals of this study were: (*a*) to estimate the proportion of the observed biomarkers that could be attributed to active smoking; (*b*) to determine the persistence of these markers in peripheral blood cells; (*c*) to calculate half-lives for each smoking-related marker, and (*d*) to document the variation in response to carcinogens between individuals with similar exposures. This study is a necessary step in the process of validating these markers as reflective of smoking-associated molecular damage.

Materials and Methods

Subjects and Data Collection. The subjects in this study were participants in a smoking cessation program at the Smoking Clinic at Columbia University/New York State Psychiatric Institute. After signing a consent form approved by the local Institutional Review Board, subjects were screened by questionnaire to exclude those who had not smoked 20 or more cigarettes per day for at least 1 year, as well as those who did not meet certain health criteria. Pregnant women, persons with a history of schizophrenia or cancer, current drug or alcohol abusers, persons with abnormal liver function (as evidenced by blood test), or those currently being treated with antidepressants were excluded.

In addition, a subject must have quit smoking for 8 months or more as verified by plasma cotinine (≤25 ng/ml). Of the 420 persons who gave baseline blood samples for this biomarker study, only 10% were successful at quitting smoking for 8 months or more and gave multiple blood samples. Two of the 42 successful quitters were ineligible, one due to cancer, and another was missing the baseline blood sample, leaving 40 persons who were eligible for the study. The 40 individuals were comparable to the 420 subjects in the parent population with respect to age, diet, ethnicity, and smoking history (packs per day and pack-years) but were somewhat lower in their level of nicotine dependence measured by the Fagerstrom scale (38).

This report describes the effect of smoking cessation on biomarkers in the 40 subjects with a "baseline" (or enrollment) blood sample and follow-up samples taken at 10 weeks and at 8 and 14 months after quitting smoking. At each follow-up visit, compliance with the cessation protocol was assessed by measuring plasma cotinine in subjects who stated that they had abstained from smoking. Subjects with plasma cotinine ≤25ng/ml were deemed "successes" and allowed to continue in the study. Three % of the subjects returning for follow-up had a cotinine level >25 ng/ml and were excluded from continuing in the study.

Not all subjects had assay results at all time points due to inadequate samples or specific assay requirements; PAH-DNA adducts were measured on all 40 subjects, 4-ABP-Hb adducts were measured on a subset of 16 persons, and GPA mutation frequency could be measured only on the 18 persons who were heterozygous (M/N) at the *GPA* locus. All assays were performed on coded samples.

At the enrollment interview, dietary, smoking, and occupational data were obtained, and blood samples (40 ml) were collected into vacutainers containing EDTA. Before blood samples were processed, a 1-ml sample was removed for analysis of cotinine to verify smoking status, and a 2-ml aliquot was removed for GPA analysis. Blood samples were refrigerated until processed (within 24 h) to separate the plasma, RBC, and leukocytes. Separated blood fractions were immediately stored at −80°C until they were removed for analysis.

In the parent study, after enrollment, the participants were randomized to receive either a placebo or Clonidine, an α-2 agonist used to treat hypertension and reduce symptoms of withdrawal from alcohol (39), opiate (40), and cigarettes (41). No effect of Clonidine on biomarker levels has been reported. Fifty % of the 40 subjects in this report were randomized to the Clonidine treatment, but no drugs were given until after the baseline blood sample was drawn. The cohort was relatively homogeneous with respect to race, socioeconomic status, and education. Ninety % of the subjects (36 of 40) were Caucasian, 5% (2 of 40) were African-American, and 5% (2 of 40) were Asian. The median family income was $50,000/year. Seventy-seven % of the subjects had completed college, and 60% had completed graduate school or had professional training. The average age was 45 ± 11.4 (range, 22–66). Forty-five % (18 of 40) of the subjects were women.

A detailed smoking history was obtained for each participant at enrollment. The subjects smoked an average of 28 cigarettes/day (range, 20–50) and had been smoking for an average of 28 years (range, 6–45), yielding an average cumulative exposure of 33.2 pack-years (packs per day × years smoking; range, 4.5–84). Cotinine levels at baseline (before attempting smoking cessation) ranged from 46 to 532 ng/ml, with a mean value of 262 ng/ml. Subjects were interviewed to determine dietary exposure to PAHs by estimating the number of servings of charcoal-broiled beef, charcoal-broiled meat (other than beef), and servings of smoked meat and fish eaten

in the past 2 weeks. Hours of exposure to ETS at home and at work in the last 2 weeks and number of other smokers in the home was reported.

PAH-DNA Adducts by ELISA. Coded samples were assayed by competitive ELISA, with fluorescence detection as described previously (42). The assay was performed by coating 96-microwell black plates (MicroFLUOR "B"; Dynatech Laboratories, Alexandria, VA) with 0.2 ng BPDE-I-DNA (5 adducts/10^5 nucleotides). A previously characterized rabbit polyclonal antiserum (43, 44) generated against BPDEI-DNA was used at a 1:1,600,000 dilution. A standard curve was constructed by mixing 50 μl diluted antiserum with *in vitro*-modified BPDE-I-DNA (1.5 adducts/10^8 nucleotides) in carrier-nonmodified calf thymus DNA such that 50 μl contained 2–120 fmol BPDE-I-deoxyguanosine adduct in 50 μg DNA/well. Samples were assayed at 50 μg DNA/well after sonication and denaturation by boiling for 3 min and cooling on ice. Goat anti-rabbit IgG-alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) was used at 1:400 dilution. The substrate, 4-methylumbelliferyl phosphate (100 μl, 50 μg/ml 0.1 M diethanolamine, pH 9.6), becomes fluorescent after phosphate removal. Fluorescence was read on a Microfluor reader (Dynatech Laboratories). Samples were run in triplicate wells, and the median values were used to determine the percentage of inhibition. When sufficient DNA was available, the assay was repeated. Samples with greater than 20% inhibition were considered positive. For analytic purposes, samples with assays showing less than 20% inhibition were classified as "nondetectable" and assigned a value of 1 adduct/10^8 nucleotides, an amount midway between the lowest positive value and zero. Although measured antigenicity may result from multiple diol epoxide adducts, because the standard curve is constructed using BPDE-I-DNA adducts, the modification level is expressed in terms of mole BPDE-I-deoxyguanosine adduct that would cause similar inhibition.

Laboratory variability was assessed by calculating the coefficient of variation for all replicate assays results. In a prior study, the coefficient of variation was 30% (45). In this study, 69% of the samples had replicate assays, with an average coefficient of variation of 34%.

4-ABP-Hb Adducts. The assay for 4-ABP-Hb adducts was performed according to the method of Skipper *et al.* (46, 47). RBC were separated and washed 3 times with cold PBS. Packed erythrocytes (1 ml) were mixed with 4 ml of cold distilled water to induce lysis. Ghosts were removed by centrifugation at 25,000 × *g* for 30 min at 0°C using a Ti 60 rotor in a Beckman ultracentrifuge at 20,000 rpm. Supernatants were dialyzed against 1 liter of distilled water at 4°C for 24 h, changing the water frequently to remove unbound 4-ABP. Total volumes and Hb concentration were measured. Two internal standards, 25 pg from 1 ng/ml stock solutions of 4′-fluoro-4-ABP and D_9-4-ABP were added, and the Hb made 0.2 M using 10 M NaOH before incubation at room temperature for 3 h. Each sample was then extracted with hexane. The emulsion was broken by centrifugation and freezing at −78°C for at least 1 h. HCl (2.5 ml, 1 M) was added to each of the hexane extracts, samples were vortexed for 1 min and centrifuged, and the hexane layers were discarded. The aqueous layers, after adjustment to pH 11 with 250 μl of 10 M NaOH, followed by the necessary amount of 1 M NaOH, were extracted twice with a total volume of 7 ml of hexane. The two extracts were combined and evaporated under nitrogen to approximately 250 μl. To 250 μl of the sample in hexane, 10 μl of a 10% v/v of pentafluoropropionic anhydride in hexane was added. Samples were vortexed for 1 min and allowed to stand for 30 min at room temperature. To 250 μl of hexane were added 250 μl of 1 M potassium phosphate buffer (pH 7), and the samples were vortexed for 1 min and allowed to stand for 1 min. The hexane layers were removed after freezing at −78°C and evaporated to dryness in small conical tubes. Samples were dissolved in 10 μl of toluene, and 1.5–2 μl was injected onto a Hewlett-Packard 5880 gas chromatography-mass spectrometer with a Chrompack C8-Wax 52 CB 10 m, 0.25 mm internal diameter, with a column thickness of 0.1 μm (using ultrapure helium carrier gas with hot on-column injection. Ions monitored were 295 (PFP-ABP), 313 (PFP-4′-fluoro-ABP) and 304 (PFP-D_9-ABP). Peak areas were integrated using standard Hewlett-Packard software, and data were converted to fmol/mg Hb using a spreadsheet.

In addition to the internal standards, a pooled sample of Hb from 50 heavy smokers was prepared as a positive control and was run with each batch of 16 samples. Batch-to-batch variation was determined by assessing the variation in the adduct levels for the aliquot from the pooled sample on different days; the coefficient of variation of the assay was 20%. When the value of the pooled control sample was outside that range, the samples were reinjected. In this analysis, each sample was injected only once.

GPA Mutation. Cell fixation, immunolabeling, and flow cytometric analysis procedures for the GPA assay were performed as described previously (48). Briefly, blood samples are first serotyped for the M/N blood group using commercial anti-M and anti-N sera (Ortho Diagnostic, Raritan NJ). Samples from heterozygous $GPA^{M/N}$ donors are fixed with formalin, then immunolabeled with two mAbs specific for the GPA^M (6A7) and GPA^N (BRIC157, International Blood Group Reference Laboratory, Elstree, Herts, United Kingdom) allelic forms of the GPA protein. Distinguishable green (BRIC157-fluorescein) and orange (6A7-biotin + streptavidin R-phycoerythrin conjugate; Caltag Laboratories, Inc. South San Francisco, CA) fluorescent labels are coupled to the antibodies so that normal erythrocytes, which express both the GPA^M and GPA^N alleles, are doubly labeled. The two fluorescence intensities of each of 5×10^5 cells per sample are quantified using a Becton Dickinson FACScan flow cytometer. Erythrocyes with normal expression of both *GPA* alleles bind both antibodies and are doubly labeled. A small number of variant cells are detected that bind the normal level of GPA^N-specific antibody but exhibit no binding of the GPA^M-specific antibody, indicating that these cells have lost expression of the GPA^M allele. These variants are designated allele loss "hemizygous N/0" cells. A second discrete population of rare variant cells appears to have lost expression of the GPA^M allele but express the GPA^N allele at twice the normal level. These variants are designated as allele loss and duplication "homozygous N/N" cells. V_fs for both classes of variants are directly measured by counting the number of both variant and normal cells and are expressed as variants per 10^6 cells. A typical analysis that simultaneously determines the V_f of both cell classes requires ~30 min to examine the 5×10^6 total cells at a rate of ~3000 cells/s. The coefficient of variation of the assay was 20%.

Cotinine. Levels of cotinine were measured in plasma using gas chromatography as described previously (49). The method involves liquid/liquid extraction of plasma, followed by gas chromatographic separation using a 30-m capillary column and a nitrogen-phosphorous detector operated in the nitrogen mode (49). The internal standard *N*-methyl cotinine was added to the plasma before extraction. Five-point standard curves were gen-

Table 1 Descriptive statistics of biomarkers (all subjects)

Assay	Baseline mean ± SD (n) Mimum to Maximum	10 Weeks mean ± SD (n) Mimum to Maximum	8 Months mean ± SD (n) Mimimum to maximum	14 Months man ± SD (n) Mimum to Maximum
PAH-DNA adducts/10^8 nucleotides	8.3 ± 8.8 (40) 1–43	6.0 ± 6.3 (31) 1–26	4.0 ± 4.1[a] (35) 1–14	5.2 ± 5.2[a] (25) 1–22
4-ABP-Hb (nm/mHb)	18.9 ± 8.7 (16) 4–31	8.1 ± 5.0 (16) 3–17	4.7 ± 3.5[a] (16) 1–15	4.8 ± 2.3[a] (15) 0–9
GPA N/O ($V_f/10^6$ cells)	7.6 ± 6.1 (18) 2–29	9.3 ± 6.4 (15) 1–23	6.6 ± 3.9 (16) 2–18	7.2 ± 4.6 (9) 2–15
GPA N/N ($V_f/10^6$ cells)	8.1 ± 4.1 (18) 2–15	10.1 ± 5.4 (15) 4–21	10.6 ± 5.6 (16) 3–23	7.5 ± 2.9 (9) 4–13
COTININE (ng/ml)	262.5 ± 114.1 (40) 46–532	5.0 ± 0 (35) 5–5	5.4 ± 2.2 (40) 5–19	5.0 ± 0 (31) 5–5

[a] No significant difference between biomarker values at 8 and 14 months.

erated for each analytical run, and low and high quality control samples were processed each day. The method requires "cold trapping" injection and temperature programming to achieve clean separation of the compounds of interest, and this results in an analysis time of 36 min. To facilitate productivity, an autosampler and on-line automatic data reduction system were used so that samples can be assayed during the evening hours and overnight if required. The detection limit was 10 ng/ml. Results below the detection limit were reported as 5 ng/ml, one-half the detection limit. The coefficient of variation of the assay is 5% intrarun and 6.5% interrun.

Statistical Methods. Descriptive statistics and frequency distributions were generated for each biomarker and questionnaire variable. Biomarker data were stratified by median level of age, income, education, self-reported smoking history (current cigarettes/day, years of smoking, pack-years, hours of daily exposure to passive smoking in the home and at work), plasma cotinine, dietary PAH (charcoal-broiled meat, smoked meat, or fish), occupational exposure to PAH, gender, and vitamin use. For categorical exposure variables, the Wilcoxon rank-sum test was used to test whether the biomarker levels differed by strata, and to assess the effect of treatment group (Clonidine/placebo) on biomarker levels at each time point. Correlations between biomarkers and questionnaire data were assessed using Spearman's rank correlation.

GPA mutations, PAH-DNA, and 4-ABP-Hb adduct data were log transformed to stabilize the variance and normalize the distributions for regression analysis. Multivariate analysis was used to generate a regression model for each biomarker. Common confounders such as age, gender, and variables that were hypothesized to contribute to exposure, such as dietary PAHs and ETS exposure at home and at work, were screened by the nonparametric correlation for inclusion in the model. Dietary PAH and ETS exposure data were included in regression models as continous and as dichotomous (no/yes) variables.

To determine the decline in biomarkers over time, Wilcoxon matched-pairs signed-rank tests were performed comparing baseline levels to 10-week, 8-month, and 14-month levels for all pairs and for the subset with samples at every time point.

To assess the magnitude of the decline and to generate half-life estimates, mean biomarker levels were calculated for the subset of persons who had assay results at every time point. Because the biomarker values appear to plateau by the 8-month sample, half-lives were first estimated graphically by inspection of the means from the baseline and 10-week samples. Half-life estimates were also generated using the log-transformed biomarker data from the baseline, 10-week, and 8-month samples and the linear model of the GEE SAS macro for longitudinal data analysis, adjusting for background by subtracting the minimum adduct value from the 8- and 14-month samples from each time point (50). Average half-life and 95% CIs were generated.

Results

PAH-DNA Adducts. The mean PAH-DNA adducts for all 40 subjects was 2-fold higher while smoking than after 8 months of abstinence (Table 1). The coefficient of variation reflecting differences between individuals was 106%. A similar but greater (2.7-fold) difference was observed when the means were calculated for subjects with samples at every time point (Table 2). The Wilcoxon matched-pairs signed-rank test demonstrated a statistically significant decline in PAH-DNA adduct levels after 8 months of cessation when all pairs were assessed ($P = 0.03$, 35 pairs; Fig. 1) and when the subset of pairs with samples at every time point were compared ($P = 0.03$, 15 pairs). PAH-DNA adducts declined significantly after 14 months of cessation ($P = 0.05$ when all 25 pairs were compared; $P = 0.06$ with the subset of 15 pairs).

Assuming a linear decline, the half-life of PAH-DNA adducts in leukocytes by inspection of the mean values at baseline and in the 10-week samples yielded a half-life estimate of 13 weeks (9 weeks if background was subtracted). Linear extrapolation from these two points would indicate that adduct levels in ex-smokers would reach background levels by a minimum of 16 weeks. The half-life from the regression model (using log-transformed biomarkers and exact date of baseline, 10-week, and 8-month samples) after adjustment for background was 23.4 weeks (95% CI, 10.5–36.3).

When subjects were dichotomized by the median income, higher income was associated with lower levels of PAH-DNA adducts ($P = 0.06$ by the Wilcoxon rank-sum test), but there was no significant difference by any other variable. In the

Table 2 Mean values of biomarkers over time in subsets with samples at each time point

Time since quitting smoking (wk)	PAH-DNA (adducts/10^8) $n = 15$	4-ABP-Hb (nm/mHb) $n = 15$	GPA N/O ($V_t/10^6$) $n = 7$	GPA N/N ($V_t/10^6$) $n = 7$
0	9.5 ± 7.9[a]	18.9 ± 9.0	8.4 ± 9.4	6.4 ± 3.8
10	5.8 ± 4.7	8.4 ± 5.0	12.0 ± 7.7	10.1 ± 6.7
36	3.5 ± 3.8	4.7 ± 3.5	7.2 ± 5.1	9.7 ± 5.1
62	4.8 ± 4.0	4.8 ± 2.3	7.3 ± 4.8	6.9 ± 3.1

[a] Mean ± SD.

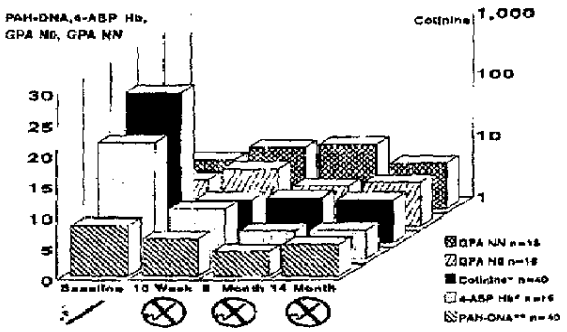

Fig. 1. Biomarkers in Smokenders (all subjects).

10-week sample, there was no significant difference in mean PAH-DNA levels measured in subjects who received placebo *versus* Clonidine (6.2 ± 7.8 *versus* 5.9 ± 4.7; $P = 0.68$). Nor was there any difference in adduct levels by treatment group at any time point.

PAH-DNA adducts both at baseline and 10 weeks after smoking were significantly associated with hours of passive exposure to ETS at home, ($r = 0.41$, $P = 0.009$, baseline; $r = 0.40$, $P = 0.02$, 10-week sample). Adducts were also significantly higher when there was another smoker in the home ($P = 0.02$). ETS at work was not significantly associated with PAH-DNA adducts at any time point.

The best multivariate regression model for predicting PAH-DNA adducts included ETS at home, age, and cotinine ($R^2 = 0.22$, $P = 0.008$), with ETS at home being the strongest predictor: standardized coefficient or β for ETS = 0.43, compared to age ($\beta = -0.25$), and cotinine ($\beta = 0.14$). Inclusion of the dietary variables did not improve the model.

4-ABP-Hb Adducts. The mean level of 4-ABP-Hb adducts at baseline was 4-fold higher than the mean 8-month value (Table 1). The coefficient of variation reflecting differences between subjects was 46%. Using the Wilcoxon matched-pairs signed-rank test, a significant decrease in 4-ABP-Hb adducts was observed by the 10-week sampling for all 16 subjects and for the 15 subjects with samples at every time point ($P < 0.01$); the decline remained significant after 8 ($P < 0.001$) and 14 ($P < 0.001$) months of cessation (Fig. 1). There was no difference in 4-ABP-Hb levels by treatment group (placebo *versus* clonidine) at 10 weeks (6.1 ± 3.6 *versus* 10.2 ± 5.5, $P = .16$) or at any other time point.

Assuming linearity, the rate of decline of Hb adducts in our study by inspection yielded a half-life of 7 and 9 weeks, with, and without adjustment for background, respectively. The regression model (using log-transformed Hb adducts at the first three time points: baseline, 10 weeks, 8 months) yielded a half-life estimate of 12 weeks (95% CI, 9.9–14 weeks). Higher order modeling is not appropriate due to limitations of the study design.

Women had higher levels of 4-ABP-Hb adducts than men at each time point, although the difference was marginally significant at baseline and 8 months after quitting ($P = 0.06$). Gender was a significant predictor of adduct levels in the sample taken 14 months after quitting smoking ($P = 0.002$). Although significantly more women reported ETS exposure at home than did men ($P = 0.04$), 8 months after quitting smoking women continued to have higher levels of 4-ABP-Hb adducts than did men after adjustment for home ETS ($P = 0.03$). These results suggest that women may be more susceptible to background exposures.

The best model for 4-ABP included current cigarettes/day, gender, and ETS at home ($R^2 = 0.25$, $P = 0.06$); the addition of dietary exposures, age, etc. did not improve the model.

GPA Mutations. At baseline the coefficient of variation for GPA N/0 mutations was 80%, for GPA N/N mutations 51%. No change in either GPA N/0 or GPA N/N V_t was associated with smoking cessation (Tables 1 and 2) in the full group (18 subjects) or in the subset (7 subjects) with samples at every time point. In fact, the mean GPA N/N V_t increased at the 10-week sampling (Table 2; $P = 0.02$ by Wilcoxon matched-pairs signed-rank test). The mean GPA N/0 and N/N V_t in the 10-week sample was not different by placebo or Clonidine treatment group (8.2 ± 7.2 *versus* 10.8 ± 5.4, $P = 0.18$; 10.2 ± 6.0 *versus* 9.9 ± 4.7, $P = 0.86$, respectively). Because no trend of GPA V_t was apparent over time, no estimates of half-life could be made for smoking-induced effects in the end point.

At baseline, GPA N/0 mutations were best predicted by either weight ($r = 0.47$, $P = 0.02$) or gender ($r = 0.40$, $P = 0.1$). Fourteen months after quitting smoking, GPA N/0 mutations were more strongly associated with weight ($r = 0.70$, $P = 0.01$), gender ($r = 0.63$, $P = 0.03$) and age ($r = 0.66$, $P = 0.01$). After cessation of smoking, a multiple linear regression model with gender and age best predicted GPA N/0 mutations ($R^2 = 0.67$, $P = 0.003$). The slope for age ($\beta = 0.03$) was small compared to gender ($\beta = 0.79$). GPA N/N mutations were not predicted by any of the variables tested.

Cotinine. The average plasma cotinine was 262 ng/ml at enrollment, had declined to 5 ng/ml by the 10-week sample ($n = 35$, $P < 0.00001$), and remained between 5.0 and 5.4 ng/ml at all subsequent time points (Fig. 1). Plasma cotinine was correlated with self-reported current cigarettes/day, ($r = 0.54$, $P = 0.0003$), but mean cotinine levels were not significantly associated with passive smoking exposure at home ($r = 0.014$, $P = 0.93$) or at work ($r = -0.142$, $P = 0.38$). In baseline samples, cotinine was more strongly correlated with 4-ABP-Hb adducts ($r = 0.40$, $P = 0.12$) than with PAH-DNA adducts ($r = 0.15$, $P = 0.33$) and was not correlated with GPA N/0 ($r = -0.05$,

$P = 0.83$) or GPA N/N variant frequency ($r = -0.007$, $P = 0.97$).

Discussion

DNA adducts have been postulated to be a result of both recent and long-term exposures from mainstream and sidestream smoke, as well as other sources of PAH in the ambient air and food. Studies have not consistently found that PAH-DNA adducts were higher in smokers than in nonsmokers (10, 11, 51). However, none of these studies was optimally designed to determine smoking specificity by monitoring the same individual after removal of exposure, and large interindividual variation in response to carcinogens may explain why it has been difficult to observe significant differences between smokers and nonsmokers in prior cross-sectional study designs. This is the first report where adducts have been shown to be smoking related due to removal of exposure in a prospective study.

We observed a 50% decline in PAH-DNA adduct levels by 1 year after quitting smoking. The remaining adducts may be due to background exposures and/or to the presence of smoking-related adducts in the longer-lived lymphocytes, which can persist for many years. After only 1 year of follow-up, it is not possible to differentiate between unrepaired damage to long-lived WBC and background exposures. Therefore, the fraction of PAH-DNA adducts attributable to smoking could be >50%. If only one-half of PAH-DNA adducts result from smoking, confounding by other sources of PAH will reduce the practical application of this assay in leukocytes to detect smoking-related damage in persons with low level exposures and may encourage the use of leukocyte subfractions.

The PAH-DNA adduct levels observed in this study after quitting smoking are in agreement with the levels found in other nonsmoking subjects analyzed by the same laboratory during the same time point (5.1 ± 4.4 adducts/10^8; Ref. 45), and the ratio of adducts in active smokers to ex-smokers is in agreement with the ratio found in our earlier study of lung cancer (51).

The persistence of PAH-DNA adducts in leukocytes from this study is markedly longer than that estimated from a pilot study of four Finnish foundry workers who were sampled after 1 month of vacation and 6 weeks later after returning to work (42). Persistence of adducts in heavy smokers is likely to be influenced by the body burden of PAH, with tar deposits or adipose tissue acting as a significant source of exposure to peripheral blood cells once smoking ceases. Hence, adducts could persist in a given group of leukocytes for a longer time period than would be expected based on the lifetime of the cells, due to a mobilization of BaP and generation of new adducts.

The half-life of the DNA adducts in rat tissues was estimated to be 17 days in peripheral blood cells, 15 days in liver, and 22 days in lung tissue (52). Extrapolations from the rat data to humans, adjusting for physiological time (Refs. 53, 54; observed time/body weight$^{1/4}$, assuming an average rat weighs 0.2 kg, and a human 70 kg) gives an estimated half-life for DNA adducts in peripheral WBC in humans of 10 weeks. This estimate is on the lower bound of the 95% CI of the half-life observed in the present study.

In addition, half-life estimates could be altered by background exposures from sources including air pollution or diet, which may vary considerably from one location to another. High levels of ambient air pollution have been associated with PAH-DNA adducts in Polish residents living in a polluted urban area (55). Similarly, PAH-DNA adducts have been associated with charcoal-broiled foods in some studies (56, 57), although not in others (51, 45). The lack of a significant relationship between PAH-DNA and data on dietary exposure to smoked or broiled meat in this study is in contrast to the results of Rothman *et al.* (56, 57) and could be due to differences in study designs and questionnaires. Our instrument did not elicit the degree or method of cooking, and misclassification of exposure could have reduced our ability to detect an effect.

The significant association between PAH-DNA adducts and ETS at home is somewhat surprising given that, as in prior studies, continuous measures of active smoking were not significantly correlated with adducts, although they were positively associated. The narrow range of smoking exposure in this study (28 ± 10 cigarettes/day) may have hindered our ability to see a dose-response. Levels of BaP and 4-ABP are reportedly enriched in sidestream smoke as compared to mainstream smoke (58). Thus, it is plausible that ETS exposure increases the DNA damage in smokers and ex-smokers.

The coefficient of variation reflecting the difference between subjects was 106% for PAH-DNA, far greater than the 34% coefficient of variation determined for replicate assays. Thus, interindividual variation in response to these carcinogenic exposures appears to be substantial.

The significant drop (55%) in 4-ABP-Hb adducts from 18.9 nm/mHb to 8.4 nm/mHb (49.5 pg/gHb to 22 pg/gHb) after 10 weeks is additional evidence that 4-ABP-Hb adducts are highly smoking related. Our ability to detect a smoking-related decline with Hb adducts was anticipated based on earlier cross-sectional studies that reported no overlap between the levels of 4-ABP-Hb adducts in smokers and nonsmokers (16, 22), and on a prior study of individuals who attempted to quit smoking (20, 59). The 55% decline in adducts reported after 10 weeks of smoking cessation in our study is consistent with that estimated previously by Maclure *et al.* (20) and Skipper and Tannenbaum (59), who saw a 72% decline over 9–11 weeks (45.8 to 13.0 nm 4-ABP/mHb). In addition to confirming the smoking-related decline in adducts after cessation, this study was able to estimate the background level of Hb adducts (4.7 nm/mHb to 12.3 pg/gHb) for this New York City population. These findings are reasonable given the 4-month life span of the erythrocyte, and is also consistent with the mean level of adducts detected in two volunteer nonsmokers (3.6 to 6.2 nm/mHb) sampled from the same study area. The absolute numbers of adducts detected in this and in prior studies were different, but all found that background exposures contribute 18–27% of the total levels (22, 59). Although prior studies used the same internal standard, these two populations did not have comparable levels of Hb adducts at the outset of the study (20). The disagreement in absolute numbers of adducts between these estimates could be due to the relatively small number of individuals in both studies, the smoking history of the subjects (amount and type of tobacco), ethnic differences, or varying levels of background exposures.

These data also suggest that women may be more susceptible to carcinogens such as 4-ABP than are men. The gender difference could be due to physiological differences or inadequate adjustment of passive ETS exposures.

The 4-ABP assay coefficient of variation was 20%, whereas the coefficient of variation reflecting the difference between subjects while smoking was 46%, indicating that the assay variablity is less than one-half the variation observed between individuals. The apparently high specificity of 4-ABP as a marker of exposure to cigarette smoke, in conjunction with the ability to estimate average exposure over 4 months, makes 4-ABP-Hb adducts a good marker for smoking exposure assessments.

Neither GPA N/0 nor GPA N/N V_f declined after smoking cessation, indicating that GPA is not sensitive to cigarette smoke exposure. Due to the requirement for heterozygosity at the *GPA* locus for the assay and the fact that only one-half of the population is heterozygous, the GPA results are limited by the small sample size. The power in this study to detect a difference in GPA V_f between smokers and nonsmokers was estimated to be 69%, in contrast to greater than 90% power for PAH-DNA and 4-ABP-Hb adducts.

Other ongoing studies of the effect of smoking on GPA V_f suggest that smokers, in some but not all the populations examined, have higher N/0 V_f.[4] The small increase in GPA N/0 mutations with age ($\beta = 0.03$) is consistent with the literature, which reports a 2-fold increase in GPA N/0 mutations over a 70-year life span (3% per year) (60). The difference between males and females is equivalent to the increase in GPA N/0 mutations over a 24-year period. A careful examination of whether GPA variants are elevated in these smokers compared to other nonsmokers, controlling for gender and age, is under way.

This validation study demonstrates that PAH-DNA and 4-ABP-Hb adducts are smoking related and decline significantly over time after cessation of smoking. However, there was no significant correlation between adducts and quantitative measures of active smoking history (cigarettes/day and pack-years). The lack of association between adducts and cigarette dose may be due to the multiple background sources of PAH in the environment, or interindividual biologic variability.

Gritz (61) has proposed the use of biomarkers in feedback studies to encourage smoking cessation by increasing the perceived risk of cancer by the smoker, changing attitudes about smoking, and adherence to cancer prevention protocols (61). Biological markers could be used to emphasize the negative aspects of continued smoking and to show improvement when the subject quits smoking. Biological markers are currently being assessed in chemoprevention trials to determine the efficacy of retinol, β-carotene, and other agents in cancer prevention (62). The incorporation of these markers in intervention programs is a promising approach to cancer prevention (61–63).

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